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Cloning and characterization of human IC53-2, a novel CDK5 activator binding protein

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ABSTRACT

We have identified *IC53-2*, a human homologue of the rat *C53* gene from a human placenta cDNA library (GeneBank Accession No.AF217982). *IC53-2* can bind to the CDK5 activator p35 by *in vitro* association assay. *IC53-2* is mapped to human chromosome 17q21.31. The *IC53-2* transcript is highly expressed in kidney, liver, skeletal muscle and placenta. It is abundantly expressed in SMMC-7721, C-33A, 3AO, A431 and MCF-7 cancer cell lines by RT-PCR assay. Stable transfection of *IC53-2* cDNA into the hepatocellular carcinoma SMMC-7721 cell remarkably stimulates its growth *in vitro*. The above results indicate that *IC53-2* is a novel human gene, which may be involved in the regulation of cell proliferation.

Key words: *IC53-2, CDK5, p35, hepatocellular carcinoma, proliferation.*

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant diseases worldwide. It is the fifth most common cancer and ranks fourth in mortality rate, behind lung, stomach and colon cancers[1, 2]. HCC is a leading cause for cancer-related deaths in many countries, mainly in Asia and Africa[3]. HCC has been ranked second cause of cancer death in China since 1990s[4]. It appears to be rising in incidence in the United States and other developed western countries[5]. Although the major viral and environmental risk factors for the development of HCC have been determined, the molecular mechanisms that contribute to tumor progression in hepatocarcinogenesis remain unknown[6]. The proliferation of HCC cells is strictly regulated by many negative or positive mediators. Recently, several tumor suppressor genes have been clarified in some oncogenic pathways. In particular, recent data have highlighted the importance of the p53 and Rb pathways in mediation of HCC cell proliferation[7, 8]. There are some regions that display high score of loss of heterozygosity (LOH) in HCC cells. For example, the LOH region within chromosome 17p13.3 in human HCC in China, and some HCC related genes have been identified from this region[9-11]. Identification of novel molecules that regulate HCC cell proliferation could improve our understanding of the regulatory networks and lead to novel diagnosis and treatment decisions.

The eukaryotic cell cycle is controlled by a family of serine/threonine kinases called cyclin-dependent kinases (CDKs). All family members share greater than 40% sequence identity, and associate with a cyclin regulatory subunit. Cdc2 kinase in fission yeast is one of the earliest identified CDK genes, which performs rate-limiting functions in both G1/S and G2/M checkpoint transition. In mammalian cells, the homologue of cdc2, CDKs controls the entry of S

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phase and M phase by differentially interacting with Cyclin A, D and E[12]. CDK5 was initially identified by biochemical purification from bovine brain and by virtue of its close sequence homology to human CDK2. CDK5 is a proline-directed kinase that phosphorylates serine or threonine immediately upstream of a proline residue. The best known role for CDK5 is in neuronal cell differentiation. There is also evidence that links CDK5 activity to the regulation of cytoskeleton, axon guidance, membrane transport, synaptic function, dopamine signaling and drug addiction[13].

Like other CDKs, monomeric CDK5 shows no enzymatic activity and requires association with a regulatory partner for activation. The active form of CDK5 was shown to be a heterodimer of CDK5 and a 25 KDa regulatory protein[14]. The regulatory subunit was subsequently shown to be a truncated form of a 35 KDa protein now known as neuronal CDK5 activator, P35[15]. By using the yeast two-hybrid screen, several P35-associated proteins were isolated, one of which is *C53*. Chen et al[16] isolated an isoform of *C53* from a human aorta cDNA library, called *IC53*. In this study, we cloned another isoform of *C53* from a human placenta cDNA library, named it as isoform 2 of *C53* (*IC53-2*). The expression of *IC53-2* was detected by Northern blot in eight human tissues and RT-PCR in eight cell lines. *In vitro* association assay indicate that *IC53-2* can bind to p35 and p25. A preliminary study showed that *IC53-2* protein was able to stimulate the growth of human hepatocellular carcinoma SMMC-7721 cell *in vitro*. We also discuss here the biological importance of *IC53-2*.

MATERIALS AND METHODS

Cloning of full-length IC53-2 cDNA

A cDNA fragment of 2265bp (bp 564-2828 in Fig 1A) was identified from a human placenta cDNA library by expressed sequence tag (EST) direct sequencing. The cDNA was called PP1553 (GeneBank Accession No. AF217982).

By searching the public dbEST, the 5'-UTR (Untranslated Region) of PP1553 is incomplete, we carry out PCR using forward primer (5'-GGGCGGGCCACAGTCTCCAGCCT-3', bp1-23 in Fig 1A and F1 in Fig 1B) and reverse primer (5'-TCAACAGAGCACACAACTGACTCTC-3', bp1595-1619 in Fig 1A and R1 in Fig 1B) based on the EST contig to get the 5'-UTR of *IC53-2*.

Sequence analysis

Homology comparisons between species were performed using BLAST algorithm through the National Center for Biotechnology Information web server (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were made at http://www.ebi.ac.uk/clustalw/ index.html (Fig 1D).

Northern blot analysis

Hybridization was carried out on human normal Multiple Tissue Northern Blot membrane (Clontech) as suggested by the manufacturer. The *IC53-2* cDNA probe was labeled with [α-^{32}P] dCTP by rediprime II random primer labeling system (Amersham Pharmacia Biotech). Northern blot membrane were prehybridized at 68°C for 1 h in ExpressHyb hybridization solution (Clontech) with 0.1 mg/ml denatured and sheared salmon sperm DNA. Then, hybridization was performed at 68°C for 2 h in ExpressHyb hybridization solution plus the specific radiolabelled probes prepared before. Membrane washing and autoradiography were done according to the published methods[17].

In vitro association assay

cDNA encoding p35, p25 and p10 were subcloned into the eukaryotic expression vector pcDNA3.1/HA2 respectively. They are designated HA2-p35, HA2-p25, HA2-p10. *IC53-2* cDNA containing the complete coding sequence (bp 1595-2441) was prepared by PCR and subcloned into the eukaryotic expression vector pcDNA4/HisMax-TOPO (Invitrogen) and verified by sequencing. The following primers were used for PCR amplification: forward primer (5'-ATGCTGCGGTTCGTGCAGAAGCGG-3', bp1595-1618) and reversed primer (5'-TCACAGAGAGGTTCCCATCAGGTTC-3', bp2417-2441). There is a Xpress Tag on the pcDNA4/HisMax-TOPO vector, so we designated it as Xpress-IC53-2. The plasmid DNA was extracted and purified with the Qiagen plasmid purification kit. HA2-p35, HA2-p25, HA2-p10 and Xpress-IC53-2 were transcribed and translated *in vitro* by using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, Wis.) with [^{35}S] methionine (Amersham Pharmacia Biotech) in accordance with the manufacturer's instruction. Then, 10 μl *IC53-2* reaction product was mixed with 10 μl reaction product of p35, p25, p10 respectively. The 20 μl reaction product mixture was incubated in TNT buffer (20 m*M* Tris-HCl, 200 m*M* NaCl, 1% Triton X-100) with anti-Xpress monocolonal antibody (Invitrogen) and 20 μl of protein A/G PLUS agarose (Santa Cruz Biotechnology). After incubation at 4°C for 2 h, the protein A/G PLUS agarose beads were washed three times with 0.6 ml of the TNT buffer. Then, the proteins were solubilized in sodium dodecyl sulfate (SDS) sample buffer, subjected to SDS/12% PAGE, and visualized by autoradiography.

Cell culture

Eight cell lines were used in the study: human hepatocellular carcinoma cell line SMMC-7721, human normal hepatocyte cell line L-02, human umbilical vein endothelial cell-derived cell line ECV304, human epithelial cervix carcinoma cell line C-33A, human cervix carcinoma cell line HeLa, human

ovary adenocarcinoma cell line 3AO, human epidermoid carcinoma cell line A431 and human breast adenocarcinoma cell line MCF-7. These cells were incubated in 5% CO_2 at 37°C and cultured in DMEM (Dulbecco's Modified Eagle Medium) with 10% fetal bovine serum (GibcoBRL), 100μg/ml penicillin, and 100 μg/ml streptomycin sulfate.

RNA isolation and RT-PCR analysis

Total RNA from 10^7 cells was extracted using the Trizol reagent (GibcoBRL) and treated with DNase I (TaKaRa). RNA quality was checked by electrophoresis in 1.5% agarose/formaldehyde denaturing gel. cDNA was prepared from 5 μg total RNA, using the Super-Script II reverse trancriptase suggested by the vender (GibcoBRL). The *IC53-2* cDNA was amplified with Taq DNA polymerase (Shanghai Promega). The forward primer (5'-CGATGAGGGTTGGATTAGA-3', bp911-929 in Fig 1A and F2 in Fig 1B) and the reversed primer (5'-TCAACAGAGCACACAACTGACTCTC-3', bp1595-1619 in Fig 1A and R1 in Fig 1B) were designed from the specific sequence of human *IC53-2* gene (PCR product size 709bp); The expression of β-actin was analyzed as a control with forward primer (5'-GGACTTCGAGCAAGAGATGG-3') and reversed primer (5'-AGCACTGTGTTGGCGTACAG-3'). The size of PCR product is 214bp. The following PCR cycling parameters were employed: 94°C for 5 min; followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s; then 72°C for 10 min. The PCR products of *IC53-2* and β-actin were then resolved on a 2% agarose gel.

Transfection

HCC SMMC-7721 cells grown in a 35 mm cell culture dish (CORNING) were transfected with pcDNA4/HisMax-TOPO-*IC53-2* plasmid DNA or pcDNA4/HisMax-TOPO empty vector DNA by means of LipofectAMINE in conditions recommended by the manufacturer (Invitrogen). Plasmid DNA (1 μg) was dissolved in 100 μl medium without serum and mixed with 10 μl LipofectAMINE reagent dissolved in 100 μl serum-free media, and incubated at room temperature for 30 min to allow DNA-liposome complexes to form. Then, 800 μl serum-free medium was added, and the DNA-liposome solution was applied onto a monolayer of 5×10^4 SMMC-7721 cells in 35 mm cell culture dish. The cells were incubated with the complexes for 5 h at 37°C in a CO_2 incubator. Following incubation, 1 ml of growth medium containing twice the normal concentration of serum was added without removing the transfection mixture. 19 h later, the medium was removed and the cells were supplied with fresh, complete medium containing 100 μg/ml Zeocin (Invitrogen) to select the stable clones.

Cell proliferation assay

SMMC-7721 cells were plated into flat-bottomed 96-well plates at a density of 1.0 $\times10^3$ cells /well. The cells were cultured for 6 days (1 day as a time-point), and the proliferation was determined by a MTS 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium, inner salt.) assay using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay Kit (Promega). 20 μl of MTS reagent was added into each well of the 96 well assay plate containing the samples in 100μl of cultured medium and incubated for 1 h at 37 °C in a humidified, 5% CO_2 atmosphere. The absorbance at 490 nm was read using a Model-550 ELISA plate reader (Bio-Rad). A growth curve with the absorbance of the MTS assay for the conversion of MTS to formazan was directly correlated with the number of living cells in culture. The experiment was triplicate-performed independently.

Immunoblots

SDS-polyacrylamide gel electrophoresis was performed in 12% vertical slab gels. For western blot analyses, 30 μg total protein was loaded into each lane. After electrophoresis, proteins were transferred to PROTRAN nitrocellulose transfer membrane (Schleicher and Schuell). The blots were probed with a 1/2000 dilution of Anti-Xpress primary antibody (Invitrogen), followed by incubation with a 1/2000 dilution of the goat anti-mouse IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology) and detected with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

RESULTS

Cloning and identification of IC53-2

Using expressed sequence tag direct sequencing, we identified a human placenta cDNA clone, PP1553 (Length: 2265bp, bp 564-2828 in Fig 1A, GeneBank Accession No. AF217982). By searching the NCBI dbEST and human genome database, the 5'- UTR of PP1553 is incomplete, we carry out PCR using forward primer (bp1-23 in Fig 1A and F1 in Fig 1B) and reverse primer (bp1595-1619 in Fig 1A and R1 in Fig 1B) based on the EST contig and get the extended 5'-UTR, full length cDNA is 2828bp. Database searches indicated that the predicted protein is 56% and 67% identity to human *C53* and *IC53* (Fig 1B and Fig 1D) respectively. To distinguish it from *IC53*, we called it *IC53-2*. *IC53-2* has two insert sequences of 710 bp (bp 378-1087 in Fig 1A and Fig 1B) and 215bp (bp 1407-1621 in Fig 1A and Fig 1B), compared with that of human *C53*. There is a stop codon (bp 1617-1619) upstream of the ATG start codon (bp 1644-1646). The polyadenylation signal AATAAA is present at bp 2738 to 2743 (Fig 1A).

The full-length cDNA of *IC53-2* encodes a protein with 281 amino acids. The *IC53-2* protein contains one consensus phosphorylation site for CDK5 (bp 2244-2249, Fig 1A, shown in bold), which is a serine or threonine followed by a proline residue.

IC53-2 was mapped to chromosome 17q21.31

with 12 exons and spans a region about 11.3Kb (Fig 1B). The exon-intron junctions matched the GT-AG rule (Fig 1C).

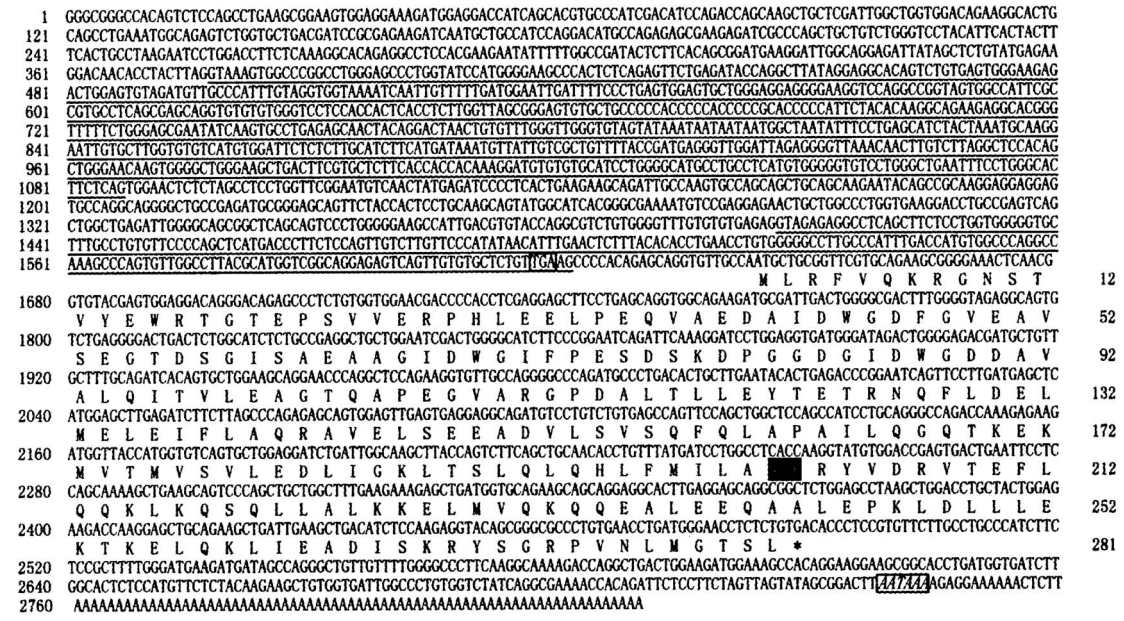
By accessing the GeneBank database for full-length cDNAs, we found that *IC53-2* had the highest sequence identity to the human *C53*, human *IC53*, rat C53, mouse *C53*, C.elegens *C53*, and fruit fly *C53* (Fig 1D).

Expression patterns of IC53-2

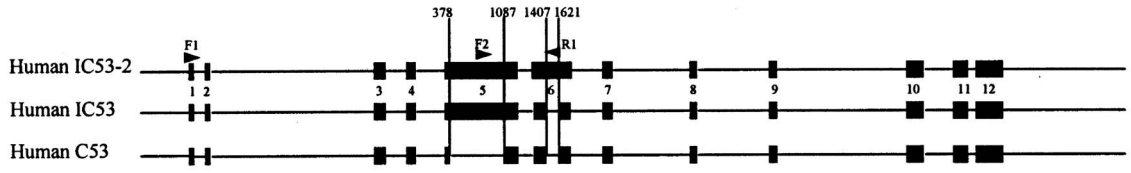
We examined the tissue expression pattern of *IC53-2* by hybridizing its cDNA probe (bp 564-2272, Fig 1A) on a multiple-tissue northern blot (MTN, Clontech). We detected two transcripts of 2.0 and 3.0 Kb in all eight normal tissues (Fig 2A).

The presence of two hybridized bands suggests that at least two isoforms exist. To confirm which

A



B



C

Gene ID	Exon No /size(bp)	5'Splice donor	Intron No /size(bp)	3'Splicer acceptor
IC53-2	Ex.1(49)	AAGATGGAGgtgtgggga	In.1(209)	tcccgccagGACCATCAG
	Ex.2(46)	AGCTGCTCGgtaggaggg	In.2(2113)	ctcatgtagATTGGCTGG
	Ex.3(132)	CTGGGTCCTgtgagtgct	In.3(280)	gctttctagACATTCACT
	Ex.4(101)	CGGATGAAGgcaagtgtg	In.4(366)	cttactcagGATTGGCAG
	Ex.5(938)	GGCATCACGgtgagcggc	In.5(176)	cctctctagGGCGAAAAT
	Ex.6(500)	GAAGATGCGgtaagatgg	In.6(698)	cctttccagATTGACTGG
	Ex.7(111)	GATTCAAAGgtgaggagg	In.7(1009)	tccttccagGATCCTGGA
	Ex.8(79)	GAACCCAGGgtaagtgca	In.8(920)	tcctggcagCTCCAGAAG
	Ex.9(89)	CTCATGGAGgtactgtca	In.9(1675)	tgtcctcagCTTGAGATC
	Ex.10(206)	CTCACCAAGgtctggctt	In.10(364)	cccgcacagGTATGTGGA
	Ex.11(172)	CAGAAGCTGgtgagatgg	In.11(102)	ctctttcagATTGAAGCT
	Ex.12(336)			

D

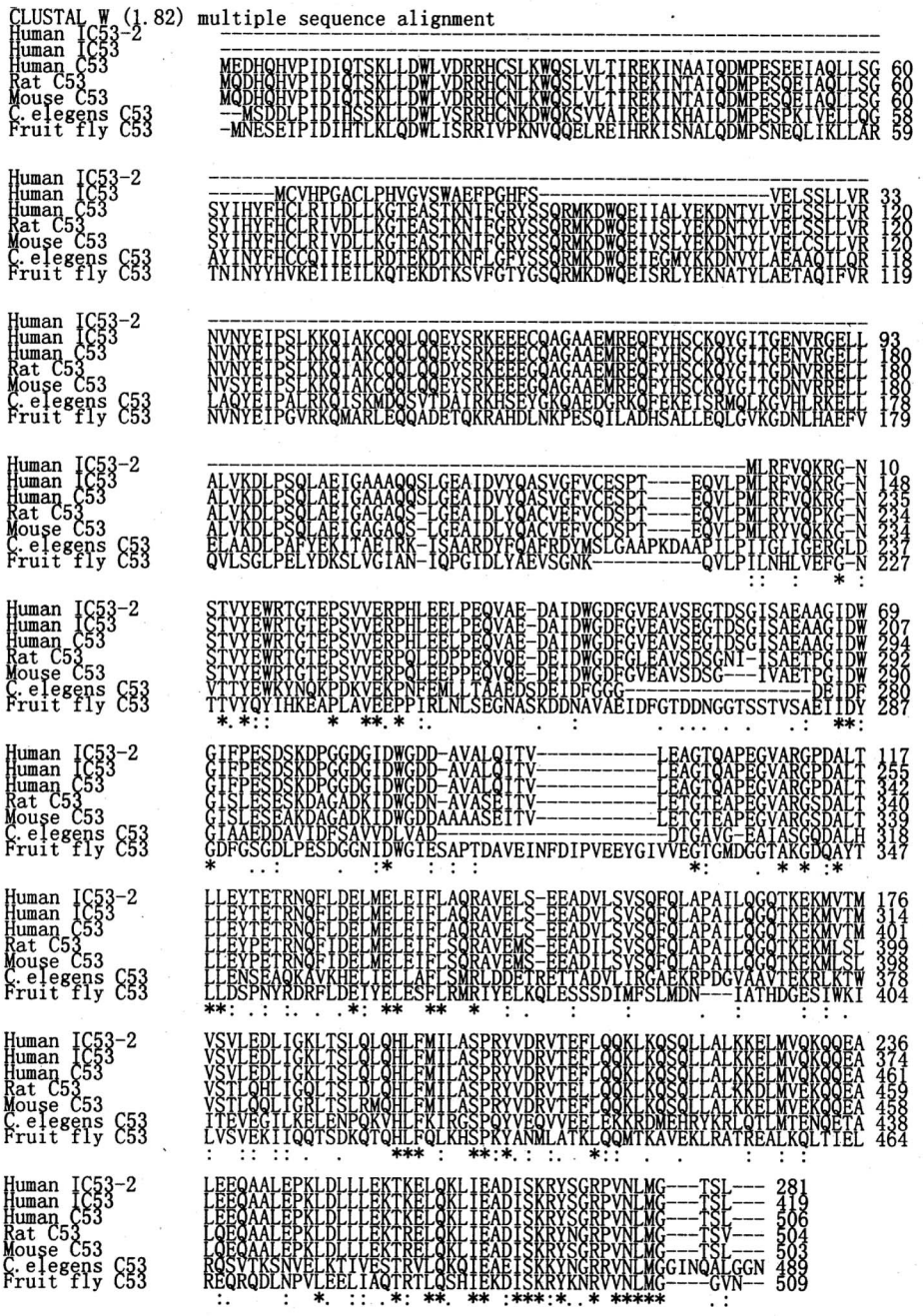


Fig 1 Nucleotide and amino acid sequences analysis of IC53-2. **A** Nucleotide sequence of human *IC53-2* cDNA and its deduced amino acid sequence. The underlined letters indicate the insert sequence compared with *C53* and *IC53* (bp 378-1087 and bp 1407-1621). **B.** Genomic structure of three transcripts of the human *IC53-2*, *IC53* and *C53* genes. Blank box indicate the exon. *IC53-2* has two insert sequences of 710 bp (from bp 378 to 1087) and 215bp (from bp 1407 to 1621), compared with that of human *C53*. **C.** Exon-intron organization of the human *IC53-2*. (Exon/intron size and nucleotide sequence at the exon and intron boundaries are given) **D.** Amino acid alignment of *IC53-2* homologues of various organisms, by clustal w program. The consensus line: "*" indicates identical or conserved residues in all sequences in the alignment; ":"indicates conserved substitutions; "." indicates semiconserved substitutions.

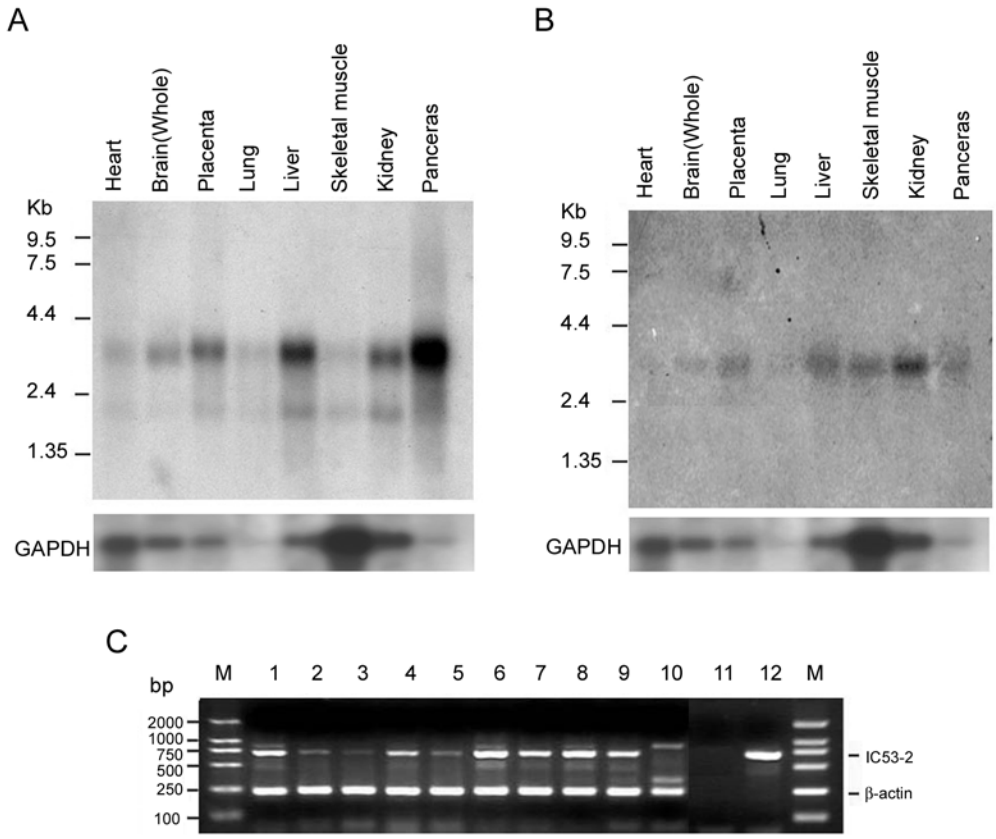


Fig 2. Tissue distribution of human *IC53-2*. **A.** Northern blot analysis using a human multiple tissue northern blot containing 2 μg poly A^+ RNA from different human tissues in each lane. GAPDH was used to evaluate the loading of mRNA. cDNA fragment of IC53-2 (bp 564-2828) was used as probe. **B.** *IC53-2* specific insert of 215bp (bp 1407-1621) was used as probe to hybridize with MTN. **C.** Expression of human *IC53-2* gene (709bp) in eight cell lines were analyzed by RT-PCR, SMMC-7721 (lane 1), L-02 (lane 2), ECV304 (lane 3), C-33A (lane 4), Hela (lane 5), 3AO (lane 6), A431 (lane 7), MCF-7 (lane 8). Human placenta cDNA (lane 9) and human normal liver genomic DNA (lane 10, 886bp). pcDNA4/HisMax vector was used as negative control (lane 11) and pcDNA4/HisMax-IC53-2 as positive control (lane 12). β-actin was used as a loading control (214 bp).

transcript is the full-length cDNA of *IC53-2*, we use the specific sequence of *IC53-2* (bp 1407-1621, Fig 1A and Fig 1B) as a probe to perform Northern blot hybridization on a MTN blot membrane. Only 3-Kb transcript was detected (Fig 2B). The *IC53-2* transcript is highly expressed in kidney, liver, skeletal muscle and placenta.

We used specific primer of *IC53-2* (forward primer: bp911-929 in Fig 1A and F2 in Fig 1B; reverse primer: bp1595-1619 in Fig 1A and R1 in Fig 1B) to examine its expression in eight cell lines by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), with β-actin as a control. Cell culture and RNA isolation were described previously. Expression of *IC53-2* was detected in all cell lines and the expression level was high in hepatocellular carcinoma (HCC) SMMC-7721 cell line, human epithelial cervix carcinoma cell line C-33A, human ovary adenocarcinoma 3AO, human epidermoid carcinoma cell line A431 and human breast adenocarcinoma MCF-7, but the expression level was low in human normal hepatocyte L-02, human umbilical vein endothelial cell-derived ECV304 and human cervix carcinoma HeLa cell lines.

IC53-2 interaction with p35 and p25 in vitro

In previous study[12], *C53* can interact with p35 through yeast two-hybrid assay. To further charac-

terize the interaction between p35 and *IC53-2*, the N-terminal (p10) and C-terminal (p25) regions of p35 were tested for their ability to bind to *IC53-2*. We found that *IC53-2* interacted with the fragment encoding the C-terminal p25 protein and not the N-terminal region p10 (Fig 3).

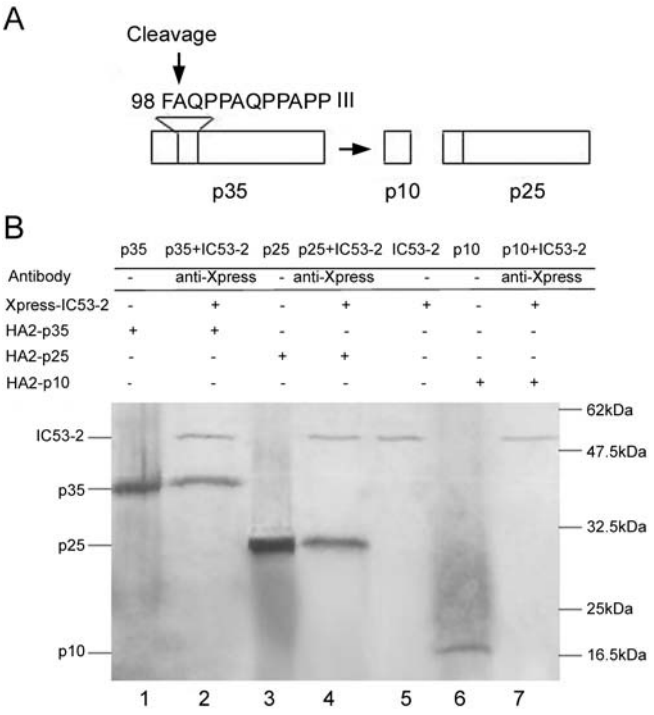


Fig 3. Interaction between IC53-2 and p35. **A.** The CDK5 activator p35. Proteolytic cleavage of p35 between residues 98 and 99 liberates the C-terminal fragment termed p25 and the N-terminal p10. **B.** IC53-2 interaction with p35 and p25 but not p10 *in vitro*. IC53-2 (lane 5), p35 (lane 1), p25 (lane 3), p10 (lane 6) were expressed by an in vitro transcription and translation reaction. Then IC53-2 was mixed with p35 (lane 2), p25 (lane 4), p10 (lane 7), followed by immunoprecipitation with anti-Xpress antibody. The protein-antibody complexes were incubated with protein A/G PLUS agarose beads, washed extensively, and boiled in SDS sample buffer. The eluted proteins were fractionated by SDS-PAGE and then subjected to autoradiograph. Molecular masses (in KDa) are indicated on the right.

Overexpressin of IC53-2 can stimulate the growth of hepatocellular carcinoma SMMC-7721 cells

From our RT-PCR assay, we detected *IC53-2* was highly expressed in SMMC-7721 cells. To characterize the effect of overexpression of *IC53-2* on cell growth, we constructed stable cell lines by transfecting *IC53-2* into hepatocellular carcinoma (HCC) SMMC-7721 cells. We used immunoblot to detect the expression of *IC53-2* in cells transfected with pcDNA4/HisMax-*IC53-2*, pcDNA4/HisMax empty vector and SMMC-7721 cells with anti-Xpress primary antibody (Fig 4B). We could see that *IC53-2* was overexpressed in the cells transfected with pcDNA4/HisMax-*IC53-2*. The cell growth curve revealed that the cells transfected with *IC53-2* cDNA had a much higher proliferating rate compared with empty vector controls ($p<0.05$). The results were reproducible and indicated that *IC53-2* was able to stimulate the growth of the cancer cells.

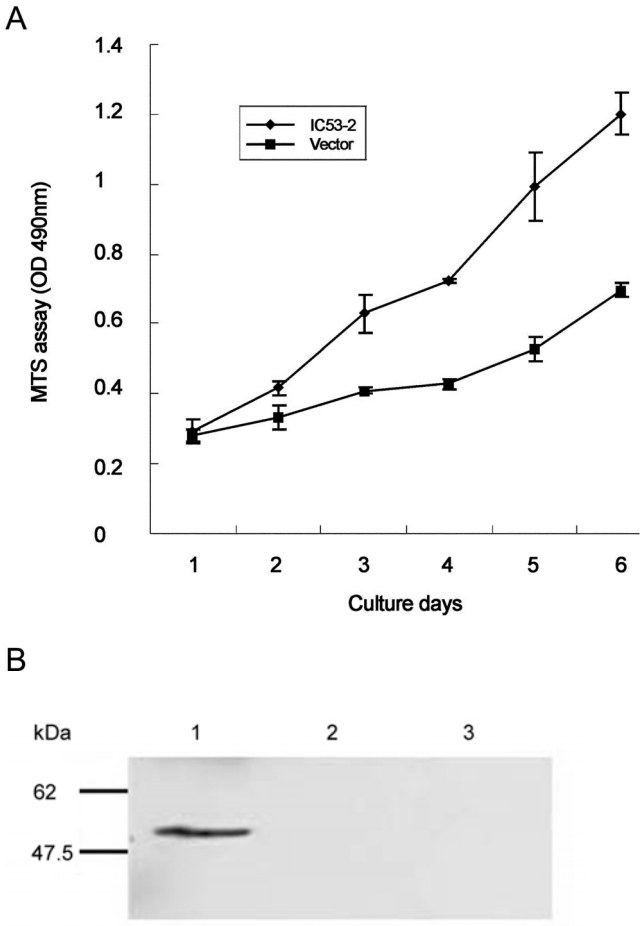


Fig 4. Cell proliferation assay of IC53-2. **A.** Cell proliferation assay of transfected HCC SMMC-7721 cells. The cell growth was examined everyday for a period of 6 days after plating. The results are means ±SD in triplicate. **B.** SMMC-7721 cells transfected with IC53-2 were assayed by western blot using anti-Xpress primary antibody (lane 1). SMMC-7721 cells transfected with empty vector (lane 2) and SMMC-7721 cell (lane 3) were assayed as control.

DISCUSSION

In this study, we have cloned and characterized *IC53-2*, a previously unknown human homologue of rat *C53* distinct from the human *C53* and *IC53*. *IC53-2*, *C53* and *IC53* are different splicing isoforms which are mapped to 17q21.31. The detail genomic structures of the three isoforms are indicated in Fig 1B. *IC53-2*, *C53*, *IC53* full-length cDNA had 2828bp, 1841bp or 2538bp encoding 281aa, 506aa and 419aa respectively. *IC53-2* mRNA is expressed in all tested cell lines and tissues and overexpressed in some tumor cell lines, such as epithelial-like hepatocellular carcinoma SMMC-7721. This result provides a clue linking the gene to the regulation of some cancer cell growth.

To determine the effects of *IC53-2* on hepatocellular carcinoma cells, we transfected *IC53-2* cDNA into the human SMMC-7721 cells and found that over-expression of *IC53-2* can remarkably stimulate SMMC-7721 cell growth. Overexpression of *IC53-2* was tested in immunoblot. Chen et al[16] detected stable transfection of *IC53* stimulates ECV304 cell proliferation by 2.1-fold compared to cells with empty vector. So *IC53-2* and *IC53* may have similar function in different tissues. In this study, we have identified high expression of *IC53-2* in some tumor cells. *IC53-2* is able to stimulate SMMC-7721 cell proliferation *in vitro*. So, we speculated high expression of *IC53-2* have positive effect on liver cancer cell growth. This paper is the first to report the overexpression and the function of human *IC53-2* in human HCC cells. Our data suggest that increased expression of *IC53-2* may play a role in multistage carcinogenesis.

The p25 protein is a cleavage product of p35 that activates CDK5 kinase robustly but lacks the amino-terminal 98 residues of p35 necessary for membrane targeting. Conversion of p35 to p25 causes prolonged activation of CDK5. Moreover, expression of p25/CDK5 complex in cultured primary neurons induces cytoskeletal disruption and apoptosis[18]. IC53-2 can bind to the fragment encoding the C-terminal p25 protein and not the N-terminal region p10. *IC53-2* and its isoforms may co-localize with CDK5 in the cells through their association with p25. Our results suggested that the interaction between IC53-2 and p25 may regulate the formation of the p25/CDK5 macromolecular complex, then negatively regulate the kinase activity of p25/CDK5 and inhibit the apoptosis of cells. During evolution, protein primary sequences and structures are often better conserved across species than nucleotide sequences[19]. The high degree of conservation indicates that *IC53-2* may play a role in an evolutionarily conserved pathway.

In conclusion, our results indicated that *IC53-2* can interact with the C-terminal of p35 and may play a role in regulating the activity of p25/CDK5 kinase. Overexpression of *IC53-2* may contribute to the liver cancer cell growth and lead to some pathological conditions in live cancer. Manipulation of *IC53-2* would have some clinical relevance to diagnose and treat liver cancer. The molecular mechanism of the growth-stimulating effect of *IC53-2* on HCC cells remains to be elucidated. Moreover, studies aiming at finding the involvement of known signal transduction pathways in such growth regulation are currently in progress.

ACKNOWLEDGEMENTS

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